THE REGULATION OF FATTY ACID BIOSYNTHESIS

Simple procedure for the purification of acetyl CoA carboxylase from lactating rabbit mammary gland, and its phosphorylation by endogenous cyclic AMP-dependent and -independent protein kinase activities

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1. Introduction

A number of key regulatory enzymes in eukaryotic cells are now known to be controlled by phosphorylation–dephosphorylation mechanisms, and there is increasing evidence that these interconversion reactions underlie the short term regulation of metabolism by external physiological stimuli (reviewed [1]).

The rate at which cytoplasmic acetyl CoA is converted to long chain fatty acids is stimulated by insulin in fat cells [2] and inhibited by glucagon in hepatocytes [3]. Acetyl CoA carboxylase has been implicated as the enzyme at which these hormonal effects are exerted, but the mechanisms are not understood in molecular terms.

Acetyl CoA carboxylase is completely dependent on the allosteric activator citrate for activity, and it is inhibited by very low concentrations of palmityl CoA [4]. There is also preliminary evidence which indicates that acetyl CoA carboxylase may be regulated by a phosphorylation–dephosphorylation mechanism. Relatively crude preparations from rat liver [5] or bovine mammary gland [6] undergo a time-dependent inactivation if they are incubated with magnesium ions and [γ-^{32}P]ATP [7–9] and ^{32}P-radioactivity is incorporated into a protein(s) which is precipitated by antiserum raised to a proteolytic fragment of rat liver acetyl CoA carboxylase [7–9]. Furthermore the inactivation can be reversed by incubation with a protein phosphatase preparation partially purified from hen oviduct [9].

If isolated fat cells are incubated with ^{32}P, a protein with a subunit molecular weight corresponding to acetyl CoA carboxylase becomes labelled, and this protein can be precipitated using a monospecific antibody raised to acetyl CoA carboxylase from rat mammary gland [10]. This experiment shows that the enzyme is phosphorylated in intact cells.

We describe here an extremely simple method for the purification of large amounts of undergraded acetyl CoA carboxylase and demonstrate that this preparation can be phosphorylated by two distinct protein kinase activities that are present as trace endogenous contaminants in the preparation.

2. Materials and methods

Phosphorylase kinase was purified to homogeneity from rabbit skeletal muscle [11] and the catalytic subunit of cyclic AMP-dependent protein kinase was highly purified from rabbit muscle by chromatography on CM–Sephadex [12]. The specific protein inhibitor of cyclic AMP-dependent protein kinase was obtained as a byproduct of the purification of protein phosphatase inhibitor-1 from rabbit skeletal muscle [13] and further purified by gradient elution from DEAE-cellulose at pH 5.0. It was enriched about 20 000-fold over the muscle extracts. Fatty acid synthetase was purified to homogeneity from lactating rabbit mammary glands by a new procedure [14]. Acetyl

CoA and NADPH were purchased from Sigma, [γ-^{32}P]ATP from the Radiochemical Centre and polyethylene glycol 6000 from BDH Chemicals.

Acetyl CoA carboxylase was assayed by a coupled spectrophotometric assay using purified fatty acid synthetase [15]. One unit of enzyme was that amount which catalysed the formation of 1.0 μmol malonyl CoA/min at 37°C, which is equivalent to 2.0 μmol NADPH oxidised/min in the coupled assay. Protein concentrations were determined by the method in [16] and in [17] using bovine serum albumin (BDH Chemicals, fraction 5) as the standard, and assuming an absorbance index, $A^{1\%}_{280\,nm}$, of 6.5. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate [18] was carried out as in [11].

The phosphorylation of acetyl CoA carboxylase was carried out at 25°C after dialysing the purified enzyme against 25 mM sodium glycerophosphate–1.0 mM EDTA–15 mM mercaptoethanol, pH 7.0, and the incubation contained 10 mM sodium glycerophosphate, 0.4 mM EDTA, 0.1 mM EGTA, at pH 7.0. Other additions to the incubation are given in the figure legends. The incorporation of radioactivity into the protein was measured after precipitation with trichloroacetic acid [19]. For the determination of phosphorylation stoichiometries the subunit molecular weight of acetyl CoA carboxylase was taken as 240 000 (see section 3) and the protein concentration was the average of the values obtained by the procedure in [16] and in [17]. These two methods gave the same value for the protein concentration within experimental error, and were also in agreement with the protein concentration determined refractometrically in the analytical ultracentrifuge [11,20] to ±10%.

3. Results

3.1. *Purification of acetyl CoA carboxylase*

Lactating New Zealand White rabbits (15–20 days post partum) were killed by an intravenous injection of pentobarbitone and exsanguinated. The mammary tissue was chopped finely with scissors, rinsed with ice cold 0.25 M sucrose–1.0 mM EDTA, pH 7.0, and homogenised at 0°C with 3 vol. of the same solution containing 15 mM mercaptoethanol. The homogenate was filtered through cheesecloth and centrifuged at 4°C for 60 min at 90 000 × *g*. The supernatant was passed through glass wool to remove floating fat (step 1) and 0.64 vol. 3.6 M ammonium sulphate were added. After standing at 4°C for 20 min, the precipitate was collected by centrifugation at 4°C for 10 min at 20 000 × *g*. The precipitate was redissolved in 100 mM potassium phosphate–25 mM potassium citrate–1.0 mM EDTA–15 mM mercaptoethanol, pH 7.0 (buffer A) and dialysed for 6 h against this buffer at room temp. (20°C). All subsequent operations were carried out at room temp. The suspension

Table 1
Purification of acetyl CoA carboxylase from lactating rabbit mammary gland

	Step (ml)	Vol. (ml)	Act. (U)	Protein (mg)	Spec. Act. (U/mg)	Purificn. (-fold)	Yield (%)
1.	90 000 × *g* supernatant	710	168[a]	12 400	0.014	1	100
2.	35% ammonium sulphate precipitate	129	176	4800	0.037	2.6	105
3.	1st 3% (w/v) polyethylene glycol precipitate	23	132	90	1.5	105	79
4.	2nd 3% (w/v) polyethylene glycol precipitate	2.0	85	20	4.2	300	51

[a] The activity at this stage may be a slight underestimate due to the presence of interfering activities which reduce the NADP formed in the coupled assay (see section 2)

300 g tissue (2 rabbits) was used in this preparation. Protein was determined by the method in [16]

was centrifuged to remove insoluble material (step 2) and 0.064 vol. 50% (w/v) solution of polyethylene glycol 6000 was slowly added to give final conc. 3% (w/v). The suspension was allowed to stand for at least 3 h, then centrifuged at 20 000 × *g* for 10 min. The precipitate was resuspended in buffer A, warmed at 37°C for 10 min and insoluble material was eliminated by centrifugation at 20 000 × *g* for 2 min (step 3). The solution was again made 3% (w/v) in polyethylene glycol, and after standing for a further 3 h, the suspension was centrifuged for 5 min at 20 000 × *g*, redissolved in buffer A and stored at 0–4°C.

A summary of the purification is given in table 1. Acetyl CoA carboxylase was purified about 300-fold in an overall yield of 40–50%. The average yield was 7 mg protein/rabbit (100–140 g mammary tissue) and the procedure was completed within 24 h. The purified enzyme contained no detectable fatty acid synthetase activity.

3.2. *Criteria of purity*

The specific activity of acetyl CoA carboxylase is similar to that reported for the mammary enzyme [15]. The purified material showed one major protein-staining band when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (fig.1) and its mobility corresponded to app. mol. wt 240 000 [14]. This value is similar to recent estimates for the molecular weight of the undegraded rat liver enzyme [21]. The preparation also contained two minor components A and B with mol. wt 230 000 and mol. wt 220 000, respectively (fig.1). These are likely to be derived from the mol. wt 240 000 species by limited proteolysis, since they copurify exactly with both the major component and with the enzyme activity, if the preparation is subjected to either gel filtration on Sepharose 4B or chromatography on DEAE-cellulose. Enzyme activity and A_{280} coincide if the preparations are subjected to further gel filtration on Sepharose 4B, and the specific activity is increased by no more than 10% if this additional fractionation step is included. The preparation also sediments as a single symmetrical peak in the analytical ultracentrifuge, yielding a sedimentation coefficient of 51 S in buffer A. A detailed characterisation of the physico-chemical properties of the acetyl CoA carboxylase and fatty acid synthetase preparations will be presented [14].

3.3. *Phosphorylation of acetyl CoA carboxylase by endogenous protein kinases*

It is often difficult to remove the last traces of protein kinase activities from enzymes which are regulated by phosphorylation–dephosphorylation, and indeed cyclic-AMP-dependent protein kinase

Fig.1. Electrophoresis of purified acetyl CoA carboxylase on 4% polyacrylamide gels in the presence of sodium dodecyl sulphate. The migration is from top to bottom and the gels were stained with Coomassie blue. A and B are minor components (see section 3.2).

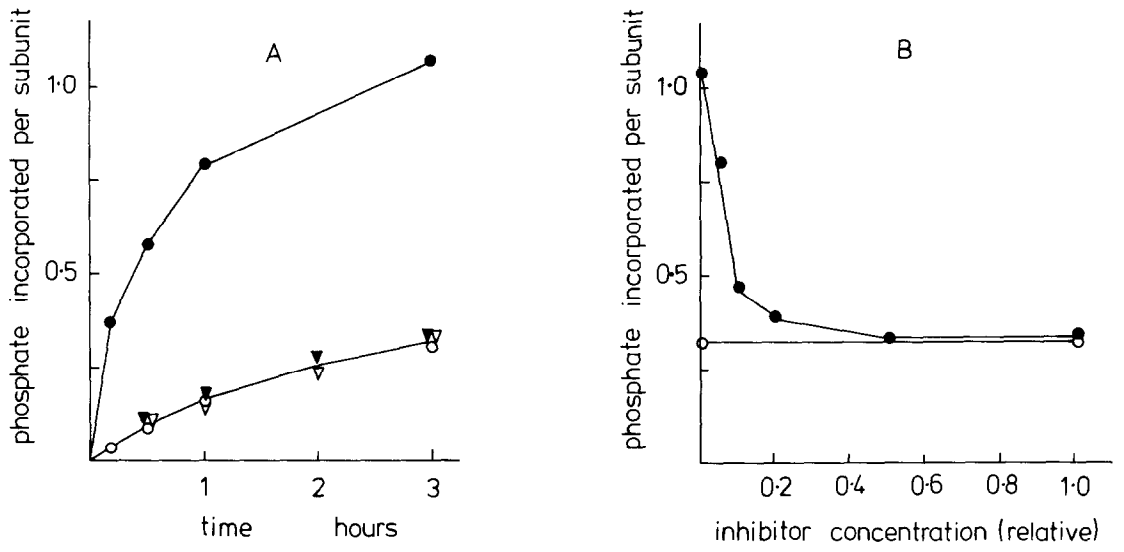

Fig.2. Phosphorylation of acetyl CoA carboxylase by endogenous protein kinases. (A) Acetyl CoA carboxylase was incubated with 10 mM magnesium acetate and 1.0 mM [γ-^{32}P]ATP in the presence (closed symbols) and absence (open symbols) of 0.01 mM cyclic AMP, and in the presence (triangles) and absence (circles) of the protein kinase inhibitor (240 U/ml). One unit of protein kinase inhibitor is that amount which inhibits 0.025 U cyclic AMP-dependent protein kinase by 50% in the standard assay [23]. One unit of protein kinase is that amount which catalyses the incorporation of 1.0 nmol phosphate into 0.2 mg mixed histone substrate/min [23]. (B) Effect of increasing the protein kinase inhibitor on the phosphorylation of acetyl CoA carboxylase in the presence (•) and absence (○) of 0.01 mM cyclic AMP. The ordinate shows the phosphorylation obtained after a 3 h incubation under the conditions described in fig.2A. The highest concentration of inhibitor used (1.0 mM) corresponds to 240 U/ml.

and glycogen synthetase kinase-2 were first identified as trace endogenous contaminants in highly purified preparations of phosphorylase kinase [22] and glycogen synthetase [23] respectively. In order to examine whether acetyl CoA carboxylase was also regulated by a phosphorylation–dephosphorylation mechanism the same experiments were therefore carried out that were used originally to identify protein kinases involved in the regulation of glycogen metabolism. The results of these experiments are illustrated in fig. 2A,2B.

When the purified enzyme was incubated with magnesium ions (10 mM) and [γ-^{32}P]ATP (1.0 mM), a slow phosphorylation of the acetyl CoA carboxylase preparation was observed, that reached a value of 0.3 molecules phosphate incorporated/subunit in 3 h (fig.2A). If cyclic AMP (0.01 mM) was also included, however, the phosphorylation of the protein proceeded much more rapidly and reached 0.5 molecules/subunit within 30 min, and just over 1.0 molecules/subunit after 3 h. These experiments showed that the acetyl CoA carboxylase preparation was contaminated with trace cyclic AMP-dependent protein kinase activity, which catalysed the incorporation of stoichiometric quantities of phosphate into the protein.

In order to investigate the phosphorylation which occurred in the absence of cyclic AMP in more detail, the experiments were repeated in the presence of a large excess of the specific protein inhibitor of cyclic AMP-dependent protein kinase. The results (fig.2A) showed that in the absence of cyclic AMP, the inhibitor had no significant effect on the rate or extent of phosphorylation of acetyl CoA carboxylase, while in the presence of cyclic AMP the phosphorylation was suppressed, but only to the same value that was observed in the absence of cyclic AMP.

An extension of these results is shown in fig.2B, which illustrates the effect of varying the protein kinase inhibitor concentration on the phosphorylation of acetyl CoA carboxylase. It can be seen that only the cyclic AMP-stimulated activity can be titrated by the inhibitor, and this activity is abolished

at the highest concentration of inhibitor used. The phosphorylation of acetyl CoA carboxylase in the absence of cyclic AMP is completely insensitive to the inhibitor.

These experiments demonstrate that the phosphorylation in the absence of cyclic AMP is not catalysed by either cyclic AMP-dependent protein kinase or its catalytic subunit, but by a protein kinase(s) whose activity is unaffected by either cyclic AMP or the protein kinase inhibitor. We propose calling this activity acetyl CoA carboxylase kinase-2, by analogy with the glycogen synthetase system [24]. Purified acetyl CoA carboxylase is therefore contaminated with traces of the holoenzyme of cyclic AMP-dependent protein kinase and acetyl CoA carboxylase kinase-2.

3.4. *Identification of acetyl CoA carboxylase as the labelled product*

In order to confirm that the mol. wt 240 000 component was phosphorylated in the experiment described in fig.2, and not another protein that might be present as a trace contaminant, polyacrylamide gel electrophoresis was carried out after phosphorylation by endogenous protein kinases under conditions in which either phosphorylation by cyclic AMP-dependent protein kinase or acetyl CoA carboxylase kinase-2 would be predominant. The results (fig.3) showed that as expected, all the radioactivity was located in the major component of mol. wt 240 000 in each case.

3.5. *Phosphorylation of acetyl CoA carboxylase and phosphorylase kinase by the catalytic subunit of cyclic AMP-dependent protein kinase added exogenously*

Acetyl CoA carboxylase and phosphorylase kinase were phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase under conditions where the phosphorylation by endogenous protein kinases was negligible (absence of cyclic AMP, very short incubation time, and low ATP and protein substrate concentrations), and the results are shown in fig.4. Acetyl CoA carboxylase was phosphorylated at 35% of the rate of the β-subunit phosphorylase kinase, which is the best physiological substrate for cyclic AMP-dependent protein kinase [25]. This rate of phosphorylation is very similar to the rate at

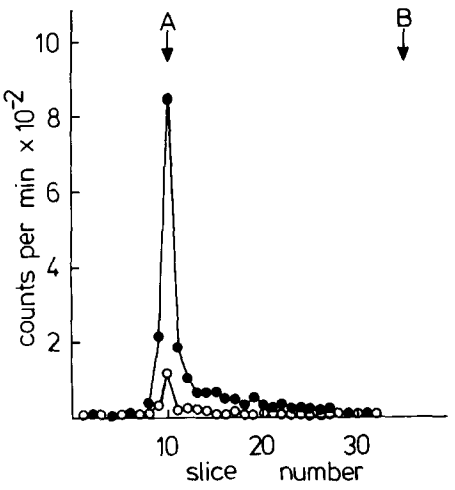

Fig.3. Phosphorylation of the mol. wt 240 000 subunit of acetyl CoA carboxylase by endogenous cyclic AMP-dependent protein kinase (•) and acetyl CoA carboxylase kinase-2 (○). The purified enzyme was incubated for 30 min with 2.0 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP and 0.01 mM cyclic AMP (•), or for 180 min with 10.0 mM magnesium acetate, 1.0 mM ATP and protein kinase inhibitor (240 U/ml) (○). Aliquots of each reaction were denatured in a buffer containing 1.0% sodium dodecyl sulphate at 100°C for 5 min, and subjected to electrophoresis on 4% polyacrylamide gels. The gels were stained with Coomassie blue, sliced into pieces of 2.0 mm in length and analysed by Cerenkov counting. The arrows A and B denote the positions of the mol. wt 240 000 subunit and the dye front, respectively.

which two other substrates of cyclic AMP-dependent protein kinase, namely L-type pyruvate kinase and protein phosphatase inhibitor-1, are phosphorylated (see [25]).

4. Discussion

The purification of acetyl CoA carboxylase described here was achieved in 1 day without the need for any time-consuming ion exchange or gel filtration chromatographies. The remarkable procedure depended on the discovery that acetyl CoA carboxylase is essentially the only protein in mammary gland that is precipitated at 3% w/v polyethylene glycol 6000. The purification factor and final specific activity are similar to the method for the rabbit mammary enzyme [15] but the

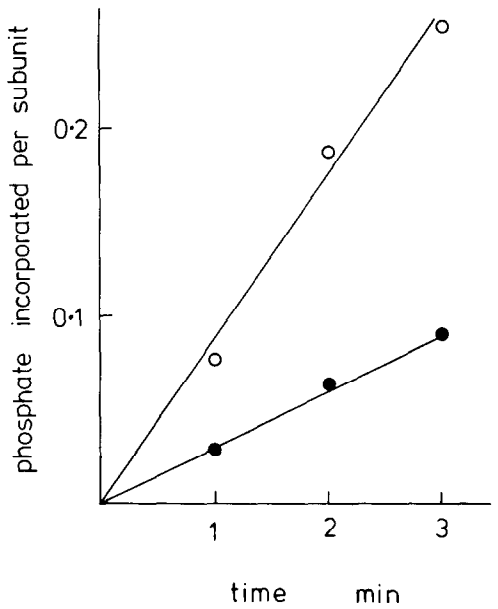

Fig.4. Relative rate of phosphorylation of phosphorylase kinase (○) and acetyl CoA carboxylase (●) by the catalytic subunit of cyclic AMP-dependent protein kinase added exogenously. The incubations were carried out in the absence of cyclic AMP at 1.6 μM protein substrate concentration, 2.0 mM magnesium acetate and 0.2 mM [γ-^{32}P]ATP. Other experimental conditions are under section 2. The stoichiometry of phosphorylation was calculated assuming a minimal binding weight of 240 000 for acetyl CoA carboxylase and 318 000 for phosphorylase kinase. Phosphorylation by endogenous protein kinase is negligible under these conditions.

yield is 20-times greater. The overall yield (40–50%) is also much higher than procedures for the rat liver [21,26] or bovine adipose tissue [27] enzymes, and the freshly prepared enzyme is almost free of the proteolytic degradation encountered [21,28].

The present work represents the first direct demonstration that acetyl CoA carboxylase is phosphorylated in vitro and that stoichiometric quantities of phosphate can be incorporated into the protein. It is also the first report that the enzyme is phosphorylated by cyclic AMP-dependent protein kinase. The finding that acetyl CoA carboxylase is phosphorylated at a similar rate to other physiological substrates of cyclic AMP-dependent protein kinase (fig.4 and section 3.5) implies that it is potentially capable of phosphorylation within seconds in vivo, and suggests that this reaction may be of physiological importance. It is tempting to speculate that this reaction might underlie the short term regulation of fatty acid synthesis by glucagon in the liver [3], assuming of course that the liver enzyme is phosphorylated in a similar manner.

The inactivation of rat liver acetyl CoA carboxylase which was observed following an incubation of the partially purified enzyme with Mg-ATP, was reported to be unaffected by cyclic AMP [5]. This suggests that the inactivation may have been catalysed by acetyl CoA carboxylase kinase-2 in these experiments. However since the protein kinase inhibitor was not included [5,7] it is also possible that the phosphorylation was catalysed by both acetyl CoA carboxylase kinase-2 and by the catalytic subunit of cyclic AMP-dependent protein kinase.

The phosphorylation of acetyl CoA carboxylase shows a striking similarity to the phosphorylation of glycogen synthetase, and raises the interesting possibility that the pathways of glycogen and fatty acid biosynthesis may be controlled in a synchronous manner. Highly purified preparations of rabbit muscle glycogen synthetase are also contaminated by endogenous cyclic AMP-dependent and cyclic AMP-independent protein kinases that phosphorylate the enzyme [19,24]. The phosphorylation of glycogen synthetase which is unaffected by cyclic AMP is catalysed by two different enzymes which have been termed glycogen synthetase kinase-2 and glycogen synthetase kinase-3 [29]. The possibility should therefore be borne in mind that 'acetyl CoA carboxylase kinase-2' is not a single enzyme.

In the case of glycogen synthetase, the current hypothesis, for which there is some evidence [1,29,30], is that the activation of cyclic AMP-dependent protein kinase mediates the inhibition of glycogen synthesis by adrenalin (muscle) and glucagon (liver), while the inhibition of the cyclic nucleotide-independent protein kinases underlies the stimulation of glycogen synthesis by insulin.

The further characterisation of acetyl CoA carboxylase kinase-2 and the effect of this enzyme and cyclic AMP-dependent protein kinase on the activity of acetyl CoA carboxylase will be described subsequently.

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